Red Meat Enhances the Colonic Formation of the DNA Adduct O^6-Carboxymethyl Guanine: Implications for Colorectal Cancer Risk

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Abstract

Red meat is associated with increased risk of colorectal cancer and increases the endogenous formation of *N*-nitrosocompounds (NOC). To investigate the genotoxic effects of NOC arising from red meat consumption, human volunteers were fed high (420 g) red meat, vegetarian, and high red meat, high-fiber diets for 15 days in a randomized crossover design while living in a volunteer suite, where food was carefully controlled and all specimens were collected. In 21 volunteers, there was a consistent and significant ($P < 0.0001$) increase in endogenous formation of NOC with the red meat diet compared with the vegetarian diet as measured by apparent total NOC (ATNC) in feces. In colonic exfoliated cells, the percentage staining positive for the NOC-specific DNA adduct, O^6-carboxymethyl guanine (O^6CMG) was significantly ($P < 0.001$) higher on the high red meat diet. In 13 volunteers, levels were intermediate on the high-fiber, high red meat diet. Fecal ATNC were positively correlated with the percentage of cells staining positive for O^6CMG ($r^2 = 0.56$, $P = 0.011$). The presence of O^6CMG was also shown in intact small intestine from rats treated with the *N*-nitrosopeptide *N*-acetyl-*N*′-prolyl-*N*′-nitrosoglycine and in HT-29 cells treated with diazoacetate. This study has shown that fecal NOC arising from red meat include direct acting diazopeptides or *N*-nitrosopeptides able to form alkylating DNA adducts in the colon. As these O^6CMG adducts are not repaired, and if other related adducts are formed and not repaired, this may explain the association of red meat with colorectal cancer. (Cancer Res 2006; 66(3): 1859-65)

Introduction

There is compelling evidence from epidemiologic data that red and processed meat intake is associated with increased risk of colorectal cancer (1). The association with red and processed meat was particularly strong in individuals eating a low-fiber diet (1). The usual explanation for these associations has been that a number of heterocyclic amines (HCA) are formed when meat is cooked and that many HCAs are known carcinogens (2). However, white meat, such as chicken, is not associated with increased colorectal cancer risk, yet chicken meat may contain high levels of HCA (2). Furthermore, variants in the *N*-acetyl gene required for the activation of HCA are not consistently associated with altered risk of large bowel cancer (3).

In 1996, we reported that red but not white meat stimulates endogenous intestinal *N*-nitrosation in humans (4). The influence of red but not white meat on fecal *N*-nitrosocompounds (NOC) excretion (measured as apparent total NOC, ATNC) has since been shown in >50 healthy volunteers, all of whom were studied in a metabolic suite where diet could be carefully controlled (4–8). The direction of an increase with increasing red meat is consistent in all individuals and can be attributed to heme iron but not inorganic iron or protein (6). At the higher levels of red meat consumption, concentrations of ATNC are of the same order as the concentration of tobacco-specific NOC in cigarette smoke (4). The majority of NOC investigated are carcinogens, but it has not been established that the NOC formed endogenously in the human gut after eating red meat are genotoxic.

Endogenous *N*-nitrosation occurs because peptides and amino acids are abundant in the intestine and can be nitrosated to form diazopeptides or *N*-nitrosopeptides (9, 10). Nitrosating agents, primarily from endogenous nitric oxide (NO) production, are also in abundant supply. O^6-methylguanine (O^6MeG) is a characteristic promutagenic and toxic adduct formed by many *N*-methyl-*N*-nitrosocompounds that either spontaneously decompose or are metabolized to intermediates which are highly reactive methylating agents that react with nucleophilic centers on DNA bases (11). In addition, various nitrosated glycine derivatives react with DNA *in vitro* to give carboxymethyl adducts [e.g., O^6-carboxymethyldeoxyguanosine (O^6CMG)] and lesser amounts of methyl adducts (e.g., O^6MeG), as shown in Fig. 1. O^6CMG has particular potential as a biological marker of DNA carboxymethylation because it does not seem to be repaired by O^6-alkylguaninealkyltransferase in *in vitro* assays (11). Nitrosation of glycine by NO under simulated physiologic conditions results in formation of O^6CMG (12).

The use of exfoliated cells as a source of markers for cancer is well established; for example, the Papanicolaou smear is universally used in cervical cancer screening. In colon cancer, the use of exfoliated cells is relatively recent but has a strong biological rationale in that exfoliated cells arise from colonic tissue itself rather than associated manifestations of cancer, such as occult bleeding (13, 14). However, isolation of exfoliated cells from fecal material is difficult, and a reliable method has only recently been developed by us (15). Using this technique, an immunohistochemical method for minichromosome maintenance proteins on recovered fecal colonocytes had 92% sensitivity for detecting distal colorectal cancers (16). In the present report, also using immunohistochemistry, we now show that the O^6CMG DNA

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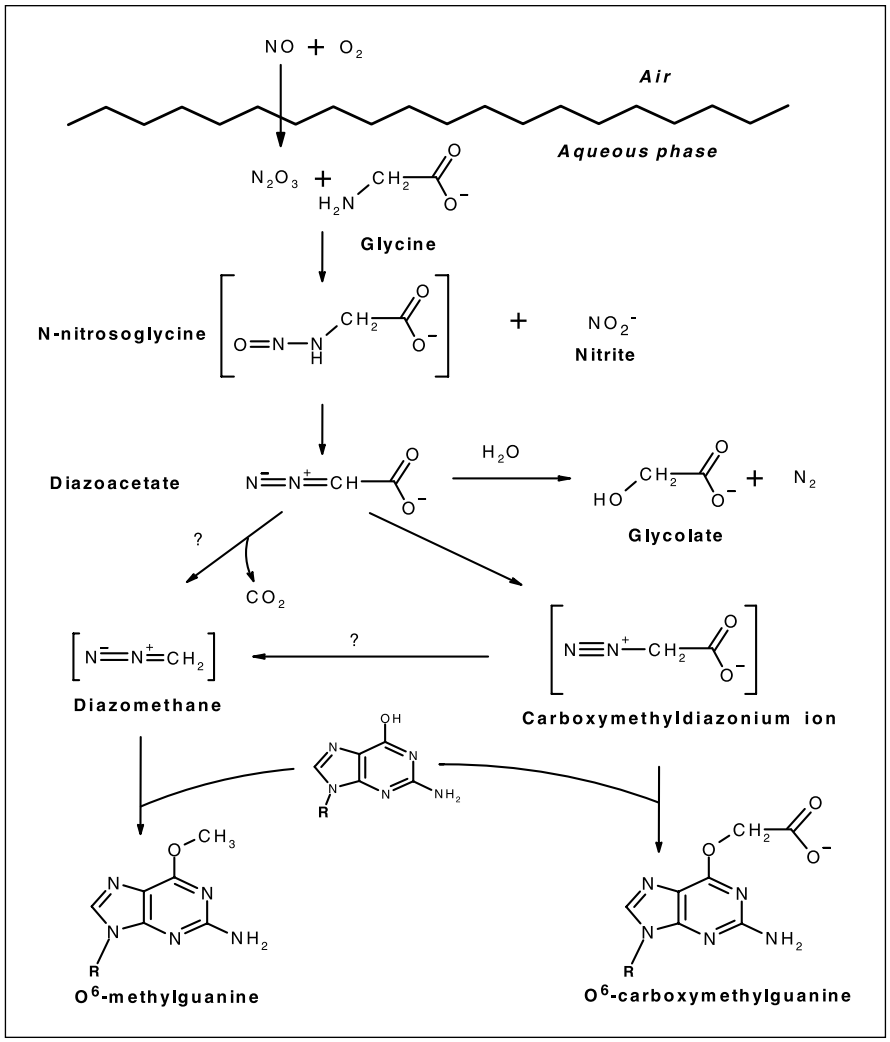

Figure 1. Summary of pathways leading to the formation of O^6-carboxymethyl and O^6-methylguanine adducts in DNA from *N*-nitrosation of glycine.

adduct is present in these exfoliated colonocytes in normal healthy volunteers, and that levels are related to NOC compounds arising from red meat consumption. We also show that the adduct is present in HT-29 cells and rat intestine treated with nitrosated glycine derivatives.

Materials and Methods

Human experimental protocol. Studies with volunteers were carried out in a metabolic suite, where all food was provided and carefully controlled, and where all specimens could be collected and processed immediately. A randomly assigned crossover design was used to avoid time effects. Each dietary period lasted 15 to 21 days, and stools for exfoliated cells were collected after volunteers had consumed each diet for 10 days. Two similar dietary protocols were followed. In the first, exfoliated cells were recovered from 8 of 12 men fed a vegetarian diet versus a 420 g red meat/day diet (6). In the second protocol, exfoliated cells were recovered from six females and seven males while they were fed a vegetarian diet (30 g fiber as nonstarch polysaccharides) versus a 420 g red meat (13 g fiber) per day and a 420 g red meat high-fiber (30 g fiber) diet per day. The subjects acted as their own control, and the diets were isoenergetic and kept constant in fat (30% total energy) by exchanging protein for carbohydrate. Basal diets contained 10 MJ/d, and individual energy requirements to maintain constant body weight were achieved using 1 MJ increments of bread, butter, and marmalade. A 3-day rotating menu was used, but the items were similar throughout to minimize day to day variation. Breakfast was composed of 40 g cereal (corn flakes, high-meat diet; Weetabix, vegetarian, and high fiber), 120 g bread (white, high-meat diet, whole-meal vegetarian, and high fiber), 20 g butter, 40 g marmalade, and a daily allowance of 300 g semiskimmed milk. The red meat was prepared to minimize HCA content, and for the evening meal, the meat was cooked in sauces given in the form of 320 g (cooked weight) minced beef pie, sweet and sour pork, and cottage pie. In a previous study with a similar menu, HCA content of the low-meat diets was 18.9 $\pm$ 4.62 μg/d, which was not significantly different from 22.1 $\pm$ 2.25 μg in the high-meat diets (7). Vegetable and pasta bake, egg and chips, and vegetable and lentil bake were given for the vegetarian evening meals. Canned fruit (100 g) and ice cream were given for dessert. Roast beef (100 g) was given as a sandwich (120 g white bread with 25 g pickle, 20 g butter, 50 g cucumber, 60 g tomato) at lunch time together with 150 g apple. Whole-meal bread and egg or cheese (40 g) were substituted for the beef on the vegetarian diet. Purified water was fed throughout, and the diets contained <13 μg preformed NOC per day (8).

Stools were collected and weighed daily, and radio opaque marker capsules were taken throughout to check compliance and for the measurement of transit time. Stool homogenates were analyzed by thermal energy analysis for ATNC, and results were given as μg/kg or μg/d after correction for daily fecal weight. Further details of techniques used in these feeding studies are reported elsewhere (4–8). The studies were approved by the Cambridge Research Committee ECS, 00/229, 04/078, 03/322.

Exfoliated cell removal. Fecal samples were excreted into plastic bags suspended on a frame below the toilet seat and immediately placed on ice

to cool for 30 minutes to 1 hour according to their size and composition. The sample was weighed and gently bathed in 1 mL/g ice cold dispersing solution (pH 7.4) comprising Eagle's medium, 5% ammonium thioglycolate (mucolytic agent), 3 mmol/L sodium butyrate, 12 mmol/L sodium bicarbonate, bovine serum albumin (10 g/L), penicillin (500 units/L), streptomycin sulfate (500 mg/L), amphotericin B (325 mg/L), and gentamicin (50 mg/L). The stool surface washing was then filtered through 125-μm and 63-μm sieves and centrifuged at 600 rpm for 5 minutes to pellet the heavier material, including exfoliated cells (15).

The cell pellet was resuspended in 15 mL cold dispersing solution, containing 5% ammonium thioglycolate, and 30 μL of FcR blocking reagent (Miltenyi Biotec, Bisley, Surrey, United Kingdom) was added. The filtrate was then mixed by rotation at 4°C for 10 minutes. Following this, 30 μL of MACS immunomagnetic beads (anti-HEA/EpCAM MicroBeads, Miltenyi Biotec) were added to the filtrate, and the tube contents were mixed for a further 20 minutes at 4°C. Bead-cell complexes were magnetically isolated by passing the filtrate through a midiMACS column (Miltenyi Biotec) attached to a strong magnet. Labeled cells remained in the column under the magnetic field and were washed twice with 3 mL PBS. After removal of the column from the magnet, the cells were gently eluted by means of a plunger in 3 mL cold PBS. The cell suspension was spun at 1,000 rpm for 5 minutes (15). In the first protocol, the pellet was resuspended with 3 mL of cold 0.1 mol/L KCl; 1 mL was frozen on dry ice and kept at −70°C for DNA isolation. The remaining 2 mL was equally divided by placing 400 μL onto five polylysine-coated slides. In the second protocol, the pellet was resuspended in 250 μL PBS; 50 μL of the cell suspension was placed onto polylysine-coated microscope slides, air-dried, and fixed using "Surgipath" cytology fixative (Surgipath Europe Ltd., Peterborough, United Kingdom). Slides were stored at 4°C before immunohistochemistry.

Immunohistochemistry of exfoliated cells. The exfoliated cells on slides were fixed in acetone at 4°C for 10 minutes then consecutively incubated with 0.05 mol/L NaOH in 40% ethanol for 5 minutes, 5% acetic acid for 15 seconds, and RNAses A (200 mg/mL) and T1 (50 units/mL) for 1 hour (at 37°C). Incubation with polyclonal O^6CMG-specific antibodies produced as earlier reported (refs. 17, 18; 1:1,000) was done overnight at 4°C. Following this stage, the standard protocol for avidin-biotin-peroxidase staining (avidin-biotin complex staining system, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and counterstaining with hematoxylin (30 seconds) were applied. Microscopic analysis was carried out at the magnification of ×1,000 (15). Exfoliated cells staining positive for O^6CMG were counted by visual scanning of the whole area of each smear and expressed as a percentage of the total cell count. In protocol two, all were stained and counted by a histopathologist (T.B.) who was blinded to the dietary period throughout. For the first protocol, one slide was stained for O^6CMG and counted by N.B. for all eight subjects. To check repeatability, a second available stored spare slide for five to seven subjects per dietary period was stained by S.T. 1 year later and counted by T.B. who was blinded to the dietary period throughout. For the second protocol, three slides for every sample were counted by T.B., and the average was taken.

Positive control preparation. Positive controls for O^6CMG identification were produced by incubating HT-29 cultured cells (American Type Tissue Culture Collection, Rockville, MD) with potassium diazoacetate (PDA; refs. 19, 20). HT-29 cells were seeded onto polylysine-coated slides and allowed to grow for 48 hours in 140-mm Petri dishes containing 50 mL of DMEM supplemented with glutamine (200 mmol/L), penicillin (100 units/L), and 10% fetal bovine serum in a humidified incubator (37°C, 95% O_2, 5% CO_2). After 48 hours, the medium was removed, and the cells were washed with 50 mL PBS. Medium containing 1 mmol/L PDA was added to the cells, and the cells were incubated for 2 hours. Following incubation, the cells were washed twice with PBS, air-dried, and fixed using "Surgipath" cytology fixative. Untreated cells were used as a negative control. Slides were then stored at −20°C until used for immunohistochemistry as described above.

Rat duodenum. Eight-week-old female Fischer rats were given a single dose (500 mg/kg) of *N*-acetyl-*N*′-prolyl-*N*′-nitrosoglycine (APNG) by gavage. Animals were sacrificed 4 hours after APNG administration. Tissue samples of duodenum were fixed in absolute ethanol and embedded in paraffin. Samples of large bowel or cecal material were not taken. Rehydrated paraffin sections of the rat duodenum were prepared and used for further immunohistochemical procedures as described above.

Analysis of K-ras mutations in exfoliated cell DNA. In the first protocol, DNA was isolated from the 1 mL suspension of exfoliated cells using a Qiagen DNA stool mini kit (Quiagen Ltd., Crawley, United Kingdom) and stored at −80°C before analysis. DNA concentration measured by absorbance at 260 nm/280 nm ranged from 0 to 30 μg per sample. All

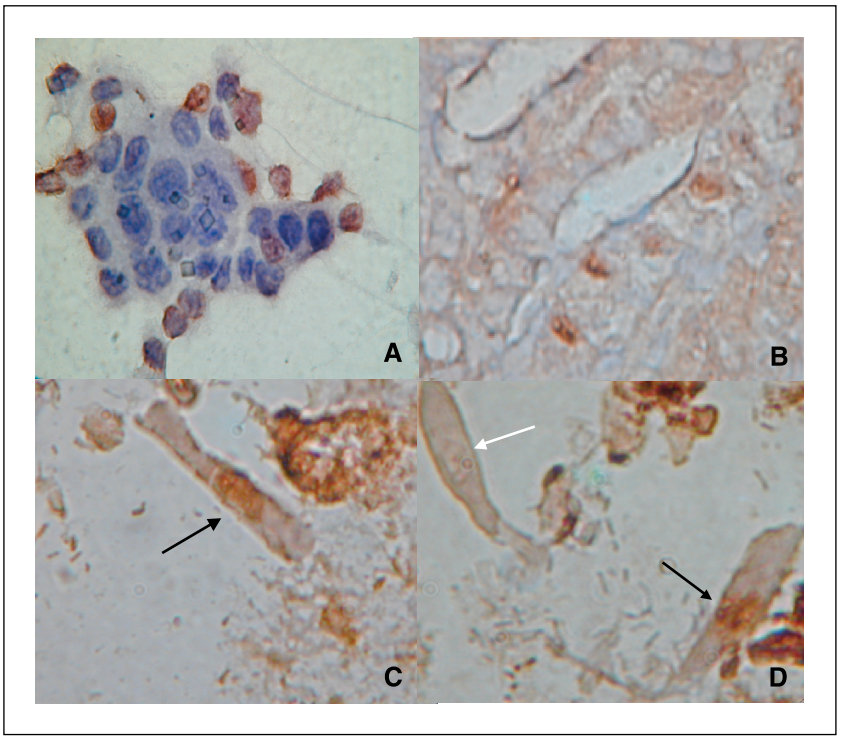

Figure 2. *A,* positive control for O^6CMG identification (HT-29 cultured cells incubated with potassium diazoacetate). Magnification, ×400. *B,* immunohistochemical detection of O^6CMG in rat small intestine. Magnification, × 400. *C-D,* presence of exfoliated colonic cells staining positive (*black arrows*) and negative (*white arrows*) for the O^6CMG adduct isolated from fecal extracts.

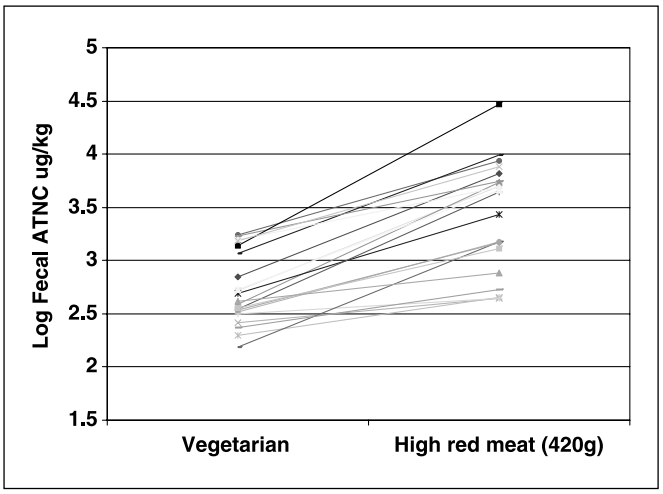

Figure 3. Change in fecal ATNC concentration on changing from a vegetarian to a high red meat (420 g) diet in 21 volunteers.

available samples were amplified using Pyromark K-*ras* assay primers for codons 12 and 13 (Biotage, Uppsala, Sweden) and 10× PCR buffer, 2 mmol/L $MgCl_2$, 1.5 units Taq Gold (Applied Biosystems, Warrington, United Kingdom), 0.125 mmol/L of each deoxynucleotide triphosphate (Amersham Biosciences, Buckinghamshire, United Kingdom), and 10 pmole of each primer. DNA (5 μL) was added to make a final volume of 50 μL. The PCR thermocycling protocol was as follows: 95°C, 5 minutes; (95°C, 15 seconds; 54°C, 15 seconds; 72°C, 30 seconds) × 45; 72°C, 10 minutes. The PCR product was captured on Streptavadin Sepharose High Performance beads (3 μL slurry; Amersham Biosciences), denatured, and washed using the Pyrosequencing Vacuum Prep workstation. The beads were then transferred to a plate containing 40 μL of 0.4 μmol/L sequencing primer, heated to 80°C for 2 minutes for sequencing primer annealing, and allowed to cool to room temperature. The templates were then sequenced using the PSQ 96MA Pyrosequencing System and genotyped using PSQ 96MA 2.1 operating software.

Statistics. Statistical analysis was carried out using Excel for Microsoft Office 2001 and SPSS version 11. In protocol two, three slides of exfoliated cells were counted from at least two samples per dietary period per subject, and the intraclass correlation coefficient was obtained in a two-way, mixed-effect model to assess the reliability of the technique. Mean and SEs and Pearson correlation coefficients are shown for the combined study results. Less than the 0.05 significance level was regarded as significant for paired two-tailed *t* testing. From previous repeat analyses on subjects on high-meat (420 g) diets, the within-person SD was 56 μg/d, and setting $\alpha = 0.05$ and β as 0.2, each study had sufficient power to detect 75 and 65 μg differences in ATNC between dietary periods with 8 to 13 subjects (6).

Results

Positive control and rat tissues. Untreated HT29 cells showed no positive nuclei, but immunohistochemical staining of cells treated with PDA for the presence of O^6CMG always revealed the presence of single cells with strongly positive nuclei and unstained cytoplasm. Figure 2*A* shows that background nonspecific staining of extracellular and intracellular structures was minimal. In the control group of untreated animals, no positive staining for O^6CMG was detected in the small intestine. In contrast, samples from all three animals of the APNG-treated group showed strong positive nuclear staining of some enterocytes (Fig. 2*B*).

Human feeding studies. Compared with the vegetarian diet, there was a highly significant increase in mean fecal ATNC concentration with red meat consumption in the 21 individuals (mean, 699 ±120 μg/kg per day for the vegetarian diet, 4,923 ± 1,387 μg/kg per day for the red meat diet). Due to the marked individual variation in response (range, 2-fold to >20-fold increase on the high-meat diet compared with the vegetarian diet), data were logged; Fig. 3 shows that the direction of response was consistent and highly significant ($P < 0.0001$). After correcting for fecal weight, mean output per day on the vegetarian diet was 193 ± 43 and 667 ± 141 μg on the high-meat diet ($P = 0.0002$). In protocol two with 13 individuals, there was a significant change in concentration of ATNC when the high-fiber, high-meat diet was consumed compared with the high red meat diet, high fiber 3,871 ± 1,093 μg /kg versus high meat 7338 ± 1973 μg /kg ($P = 0.014$) but not when corrected to output per day: high fiber 819 ± 213 versus high meat 968 ± 175 μg/d ($P = 0.447$).

There were no significant ($P > 0.05$) differences between dietary periods in mean transit time (vegetarian diet, 57 ± 22 hours; high-meat diet, 65 ± 26 hours; high-meat, high-fiber diet, 71 ± 47 hours). Daily fecal weight was, however, significantly greater on the vegetarian diet compared with the high-meat diet (vegetarian, 277 ± 126 g; high meat, 172 ± 70 g, $P = 0.001$; high meat, high fiber ($n = 13$), 243 ± 110 g, $P > 0.05$). In protocol two, an average of 3 to 120 exfoliated cells per sample was found. On paired *t* testing of means, there were no significant effects between dietary periods (vegetarian, 19 ± 4; high meat, 19 ± 4; high meat, high fiber, 12 ± 4 cells per 100 g stool; $P > 0.05$). The intraclass correlation coefficient was highly significant ($r = 0.797$, $P < 0.0001$).

Figure 2*C* and *D* illustrates exfoliated cells staining positive and negative for O^6CMG. In protocol one, for the red meat and vegetarian diets, mean percentage positive cells were 59 ± 8 and 14 ± 5, respectively, when prepared by N.B. (high meat versus vegetarian, $P < 0.01$, paired *t* test on nine subjects) and 32 ± 7 when prepared by S.T. 1 year later (high meat versus vegetarian, $P < 0.03$ unpaired *t* test on six and eight subjects). In protocol two, the mean percentage of exfoliated colonocytes staining positive for the O^6CMG adduct on the high meat diet was 56.7 ± 4.8% compared with 23.1 ± 3.5% on the vegetarian diet ($P < 0.0001$). The level of 45.7± 5.8% on the high-meat, high-fiber diet was not significantly different from the red meat diet ($P = 0.054$). The intraclass correlation coefficient was highly significant ($r = 0.753$, $P < 0.0001$).

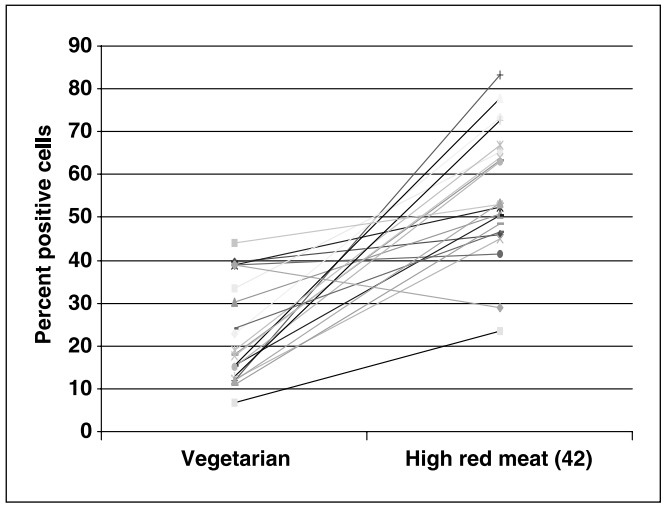

Figure 4. Change in percent cells staining positive for the O^6CMG adduct in exfoliated colonic cells isolated from fecal extracts in 21 volunteers.

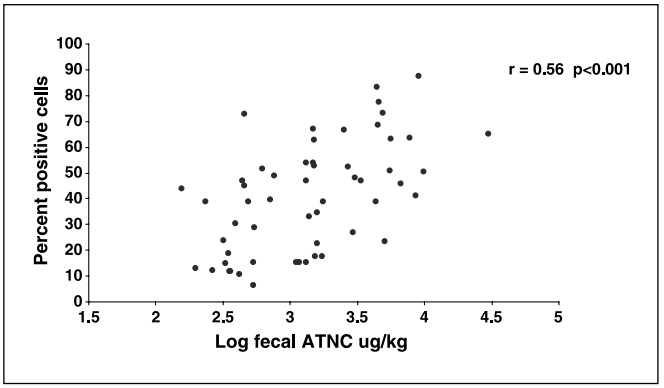

Figure 5. Correlation between fecal ATNC concentration and percent cells staining positive for the O^6CMG adduct in exfoliated colonic cells isolated from fecal extracts (n = 55).

When results from all 21 individuals from the two protocols were combined, there remained a highly significant increase in the mean percentage of exfoliated colonocytes staining positive for the O^6CMG adduct with red meat consumption compared with the vegetarian diet (55.7 $\pm$ 3.3% for the red meat diet, 22.7 $\pm$ 2.6% for the vegetarian diet; P < 0.001). Figure 4 shows the levels increased in nearly all the individuals studied.

To assess the correlation between fecal ATNC and the presence of the O^6CMG adduct, data from all three dietary periods were combined (n = 55). Figure 5 shows that levels of ATNC were consistently associated with percentages of cells staining positive for the O^6CMG adduct (r = 0.56, P < 0.001).

Successful K-ras PCR amplification was achieved in 62 of the 88 DNA isolates from exfoliated cells collected in the first protocol. No mutations in K-ras were found.

Discussion

Large bowel cancer is the second most common cancer in western countries, and nearly one million cases occur each year worldwide (21). Migrant studies, secular changes, and studies of twins all show that there are major environmental causes of colorectal cancer, and it has been estimated that up to 80% colorectal cancer can be attributed to diet (22, 23). Potentially, colorectal cancer is thus a largely preventable disease.

However, the dietary determinants of colorectal cancer have been difficult to establish. There is recent compelling epidemiologic evidence that red and processed meat are associated with increased risk, especially in individuals who consume low amounts of fiber (1), but understanding of the reason for these associations is needed before major advances in prevention of this common disease can be made.

Studies of biomarkers that are relevant to colorectal cancer, and which might provide a link between the epidemiology and the physiology of carcinogenesis in humans, are difficult due to the inaccessibility of colon tissue and the fact that there are no blood-borne risk markers in colorectal cancer. Stool DNA is widely used as a source of biomarkers for colorectal cancer detection, but the majority is bacterial DNA and estimates of the amount of human DNA as a proportion of the total vary from 0.01% to <1% (14, 24). Studies of DNA attributed to human colonocytes are likely to provide useful insights into some aspects of nutrition and carcinogenesis in human studies (e.g., methylation and folate metabolism; ref. 24). Extraction of RNA attributed to colonocytes has also been reported (25), but although bacterial and eukaryotic RNA can be distinguished, stool RNA could be contaminated with bovine RNA when high-meat diets are consumed.

Due to these difficulties with conventional stool markers, we have improved original methods for the extraction and histochemical analysis of exfoliated cells from the surface of stools (13, 26, 27). Lower volumes of washing reagents, the use of a pH stable mucolytic agent, smaller MACS microbeads to avoid interference with the visibility of cells, and a blocking agent to minimize nonspecific binding to the exfoliates considerably improved the quality of the extracted material (15). These cells are of epithelial origin, as shown by their morphology (Fig. 2*C* and *D*) and immunostaining for epithelium-specific cytokeratin C50. Relatively few cells were viable as judged by testing for trypan blue exclusion (15). There was no positive staining for apoptosis-specific antibody PAR-4 despite the occasional presence of apoptotic colonocytes visualized with picnotic nuclei. Large numbers of apoptotic cells in the colon would not be expected because apoptosis is rapid, with cells being cleared possibly in less than an hour (28). Thus, in the normal colon, only one apoptotic body is seen in every fifth crypt (29).

Using this improved technique, a new screening procedure for colorectal cancer has been proposed based on immunohistochemistry in exfoliated cells for MCM5 marker of proliferation (16). However, the use of exfoliated cells for general population screening is hampered by the short life of exfoliates once excreted. In the present study, all samples were handled within 3 to 5 hours of passing. Numbers of exfoliated cells found were orders of magnitude lower than those reportedly found in early studies of whole stool samples (27). The lining of the gastrointestinal tract is replaced every 2 to 3 days in rodents and other mammals, and the balance of cell loss has been attributed to cell shedding via exfoliation. However, there is little compelling evidence that much exfoliation into the colon occurs (28). Despite being inconspicuous in histologic material, it has been argued that apoptosis and subsequent engulfment by adjacent epithelial cells and macrophages within the crypt is the central feature of the regulation of cell number in the gut (28). There is extensive mixing in the large bowel, and even if large numbers of colonic cells are exfoliated, the majority are likely to be quickly degraded in the proteolytic environment (30). Hence, the cells detected here in fecal washes from the surface of the stool are likely to have been derived from the descending colon and sigmoid junction, where luminal contents are concentrated before stools are passed. To assess the repeatability (and therefore reliability) of the technique, at least two samples per dietary period per person were obtained, and three slides per sample were counted in protocol two, and the intraclass correlation coefficient was calculated as a measure of the between-sample variance compared with the overall variance, including variation from slide to slide. Despite the low numbers of cells present, a highly significant intraclass correlation coefficient (r = 0.797, P < 0.0001) was obtained, indicating high reliability of this technique. Although it is possible that dietary factors would affect exfoliation, we found no evidence that meat or fiber affected total cell counts. However, there was a marked effect of diet on the levels of the O^6CMG adduct (P < 0.001). In protocol two, the intraclass correlation for numbers of cells positive for the O^6CMG adduct again showed that this procedure was highly reliable (r = 0.753, P < 0.0001). Repeat measures on each sample were not available in protocol one, but when slides from protocol one were restained for the presence of O^6CMG 1 year later by a different individual, the

same effect of diet was found. Throughout, the histologist counting the slides (T.B.) was kept blind to the dietary period.

Exposure of the colonic mucosa to direct-acting mutagens and protective agents present in the luminal contents is central to current models of the etiology of colorectal cancer, particularly left-sided colon cancer, where stasis of luminal contents and contact with NOC occur (31). It is widely believed that cancers are derived from stem cells located at the bottom of the crypt. Exposure of stem cells to potential direct-acting genotoxins in luminal contents would be unlikely to occur in this case, but it has been proposed that cells on the top surface of the mucosa can be initiated and transformed, allowing "top down" clonal expansion and morphogenesis of colorectal tumors (32). From previous work, we have established that luminal contents of possible genotoxins, in the form of NOC, consistently and predictably increase when individuals are fed red meat, as is shown once more in Fig. 3. The effect of red meat is probably attributable to hemoglobin and myoglobin, which are readily nitrosated to *N*-nitrosohemoglobin and *N*-nitrosomyoglobin (33). NO has also been shown to react directly with hemoglobin and myoglobin to produce NOCs (34). More specifically, the reaction of a heme-containing mutant cytochrome *c* peroxidase with peroxide gave a product capable of oxidizing *N*-hydroxyguanidine or N^{ω}-hydroxyarginine, resulting in the NOC *N*-nitrosoarginine (35).

However, until now, there has been only circumstantial evidence that the NOCs formed in the large bowel after eating heme from red and processed meat may be important genotoxins. For example, many of the mutations found in colorectal cancer are G-A transitions, characteristic of exposure to alkylating agents, such as NOCs (36). No K-ras mutations were found in the DNA extracted from exfoliated cells obtained from these healthy human volunteers, which is as expected because mutations relevant to colorectal cancer are generally not found in stool screening assays from noncancer cases (37).

The use of adducts as markers for genotoxicity and cancer risk is well established. Selective and targeted benzo(a)pyrene diol epoxide adducts occur at the major p53 mutational hotspots in lung cancer (38), and in a prospective nested case-control study, the presence of the aflatoxin-N^7-guanine adduct in 24-hour urine samples conferred a highly significant relative risk of 7.6 (3.2-18.0) for hepatocellular carcinoma (39). Malonaldehyde DNA adducts have been detected in oral mucosa cells (40) and in the colonic mucosa (41). Human colorectal cancer tissue has long been known to contain O^6MeG and N^7-methylguanine, which arise from exposure to methylating agents, such as some NOCs. N^7-methylguanine is nonmutagenic, and O^6MeG is repaired by O^6-alkylguanine DNA transferase (MGMT) so that K-ras mutations in human colorectal cancer are associated with low activity of MGMT rather than with O^6MeG (42, 43). However, O^6CMG is not repaired and therefore could be expected to be present in greater quantities in human tissue than O^6MeG. Although previously assumed to be nonmutagenic, we have recent *in vitro* evidence that shows that O^6CMG is in fact a potent mutagen producing G-A transitions and G-T transversions in adducted p53 cDNA.[4]

We established positive controls for the O^6CMG adduct measured in exfoliated cells, by incubation of a colon cell line with the direct-acting nitrosated amino acid diazoacetate. In rats treated with APNG, adducted cells were present in the duodenal tissue. APNG has a short half-life of <2 hours and would not have reached the colon when given by gavage. Figure 1*B* shows that adducted cells were located *in situ* along the length of the villi; there is thus no evidence that the adducted cells are more likely to be adducted after they have exfoliated.

We attempted to quantitate the levels of O^6CMG adducts in the DNA extracted from the exfoliates in protocol one, but levels were below the level of detection and could not be reliably established by the most sensitive method available, the immunoslot blot technique (data not shown; ref. 12). We therefore quantitated our results by standard cell counting techniques, with the histopathologist (T.B.) blinded to the subject and dietary period. Using this technique, we were able to show a significant relation between the percentage of cells staining positive for the O^6CMG adduct and the level of ATNC in fecal homogenates (Fig. 5). Furthermore, the percentage of cells staining positive for the adduct significantly increased following the change from vegetarian to a high red meat diet (Fig. 4). Although not as consistently significant, levels on the high-fiber, high-meat diet were intermediate between the vegetarian and high-meat diet. This may account for the effect of fiber in modifying cancer risk from red and processed meat, although other protective effects of fiber are known, such as increased fecal weight leading to increased dilution, butyrate production, and decreased transit time (1, 44).

By using a noninvasive technique in healthy individuals, we have shown the genotoxicity of endogenous NOC formed in the intestine after red meat consumption in humans. The link we have shown here amongst red meat, the endogenous formation of NOC, and the resulting promutagenic adduct in cells exfoliated from the colonic mucosa offers a mechanism underlying the association between red meat consumption and colorectal cancer. Prospective studies of red meat or heme consumption in relation to O^6CMG levels and mutation analysis in tumors from patients who later develop colorectal cancer will be needed to corroborate this suggestion.

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[4] S. Ponnada et al. O^6 carboxymethyl deoxyguanosine: a potent mutagenic adduct characterised using ARMS-PCR assay following site-specific incorporation into a p53 cDNA sequence. 2006, submitted for publication.

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